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Expression of a multidrug-resistance gene in human tumors and tissues

(cancer chemotherapy/doxorubicin/vinblastine)

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ABSTRACT **The identification and cloning of a segment of a human multidrug resistance gene (*mdr1*) was reported recently. To examine the molecular basis of one type of multidrug resistance, we have prepared RNA from human tumors and normal tissues and measured their content of *mdr1* RNA. We find that the *mdr1* gene is expressed at a very high level in the adrenal gland; at a high level in the kidney; at intermediate levels in the lung, liver, lower jejunum, colon, and rectum; and at low levels in many other tissues. The *mdr1* gene is also expressed in several human tumors, including many but not all tumors derived from the adrenal gland and the colon. In addition, increased expression was detected in a few tumors at the time of relapse following initial chemotherapy. Although controlled clinical studies will be required, our results suggest that measurement of *mdr1* RNA may prove to be a valuable tool in the design of chemotherapy protocols.**

Resistance to multiple chemotherapeutic agents is a common clinical problem in the treatment of cancer; such resistance may occur in primary therapy or be acquired during treatment. To study the problem of multidrug resistance, several laboratories have isolated cell lines resistant to the vinca alkaloids, doxorubicin (former generic name, adriamycin), actinomycin D. and related agents (1–7). Decreased drug accumulation as a result of increased drug efflux is found in such cell lines (3, 8). These multidrug-resistant cells contain an amplified gene, which we have termed *mdr1*, that is transcribed into a 4.5-kilobase (kb) mRNA (9–13). The protein product of this gene is the 170-kDa membrane glycoprotein which is expressed in multidrug-resistant cell lines (1, 3, 4, 7). This glycoprotein binds vinblastine, and this binding can be inhibited by vincristine and daunomycin as well as by agents, such as verapamil, that reverse multidrug resistance (14, 15). The sequence of the human *mdr1* gene has been determined and shows the presence of transmembrane regions and nucleotide binding sites, consistent with the function of the 170-kDa glycoprotein as an energy-dependent drug-efflux pump (16).

We have described elsewhere the isolation and characterization of multidrug-resistant human KB carcinoma cell lines (6–8, 17). In collaboration with the laboratory of I. Roninson (Univ. of Illinois Medical School, Center for Genetics), we have isolated from one of these human cell lines a DNA probe for the *mdr1* gene (18). Using this *mdr1* probe, we have shown, in several multidrug-resistant cell lines and in gene-transfer experiments, a correlation between the level of *mdr1* mRNA levels and the degree of multidrug resistance (9, 19). In addition, we have shown that increased *mdr1* gene expression can occur prior to gene amplification in the development of drug resistance in tissue culture. Since expression of the *mdr1* gene is responsible for multidrug resistance in tissue culture systems, we measured levels of *mdr1* mRNA in normal human tissues and in untreated and treated human cancers. Our results show that some normal tissues and tumors express the *mdr1* gene at elevated levels. We suggest that measurement of the levels of *mdr1* mRNA may prove to be useful in guiding chemotherapy.

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MATERIALS AND METHODS

Materials. Deoxycytidine 5′-[α-^{32}P]triphosphate (3000 Ci/mmol; 1 Ci = 37 GBq) and uridine 5′-[α-^{32}P]triphosphate (3000 Ci/mmol) were from New England Nuclear. Promega Biotec (Madison, WI) was the source of pGEM4 and the Riboprobe Gemini system II, and New England Nuclear manufactured the nick-translation system. All other reagents were of the highest purity available.

Cell Lines. KB-3-1 is the drug-sensitive parental cell line. KB-8-5, which is 3 times as resistant to doxorubicin and 6 times as resistant to vinblastine, was derived in two steps from KB-3-1 (6). This cell line has increased levels of *mdr1* mRNA without gene amplification (9). Cell line KB-V1 (Vbl) was isolated in successive steps from KB-3-1; it is 420 times as resistant to doxorubicin and 210 times as resistant to vinblastine (7). It has amplified the *mdr1* gene and expresses *mdr1* mRNA at a very high level. Three neuroblastoma cell lines (HTB 10, HTB 11, and CCL 127) and a colon carcinoma cell line (HTB 38) were obtained from the American Type Culture Collection. Colon carcinoma cell lines (WIDR and LS-174) were gifts from J. Schlom (National Cancer Institute). The rat pheochromocytoma line PC12 was obtained from M. Nirenberg (National Institutes of Health).

***mdr1* Hybridization Probes.** cDNA was prepared from KB-C2.5 cells, which contain large amounts of *mdr1* mRNA, and inserted into the *Eco*RI site of bacteriophage λgt11. A simplified cDNA restriction map is shown in Fig. 1. Details of the isolation and characterization of this cDNA will be published elsewhere (23). The *mdr1* probes utilized were obtained from contiguous areas starting at the 5′ end of the *mdr1* cDNA and cover more than half of the total cDNA. Probe 10 was labeled by nick-translation before use in the slot blot analysis. Probe 5A was cloned into the *Eco*RI site of pGEM4 and used to make an RNA probe with SP6 RNA polymerase (20). This RNA probe was used in all blot-hybridization analyses of electrophoretically fractionated RNA. A β-actin cDNA was kindly provided by B. Paterson (National Cancer Institute).

RNA Extraction, Electrophoresis, and Blot Analysis. All samples were kept frozen at −70°C. Solid tumors were pulverized on a metal surface placed on a bed of dry ice, prior to RNA extraction. Frozen buffy coats from leukemia samples were thawed rapidly at 37°C and centrifuged. Total cellular RNA was extracted by homogenization in guanidinium isothiocyanate followed by centrifugation over a cesium

Abbreviation: kb, kilobase(s).

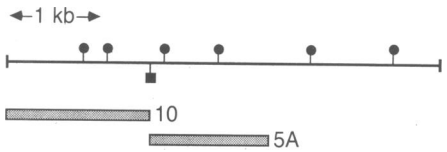

FIG. 1. Simplified restriction map of *mdr1* cDNA. Probe 10 was sed for slot blot analysis. Probe 5A was used to make an RNA probe or the electrophoretogram blot analyses. Restriction sites for *Eco*RI) and *Pvu* II (●) are shown.

nloride cushion. The RNA was electrophoresed in 1% garose/6% formaldehyde gels as described (9). Six or eight icrograms of total RNA was loaded per lane. With one xception, only samples in which the ribosomal RNA appeared undegraded were analyzed. Hybridization was in the reviously described (9) buffers for 16 hr at 55°C with 2×10^6 pm of synthetic RNA per ml. The blots were washed by ddition of 1× SSC/0.1% $NaDodSO_4$ warmed to 68°C at oom temperature every 20 min for 1 hr, followed by two)-min washes with 0.1× SSC/0.1% $NaDodSO_4$ in a 70°C ater bath. (1× SSC = 0.15 M NaCl/15 mM sodium citrate, H 7.) Autoradiographic exposures were for 2–4 days.

Slot Blot Analysis. The filters were presoaked in 10× SSC, nd then 10 μg of total RNA in 100 μl of 10× SSC was pplied. After baking, prehybridization and hybridization ere performed at 42°C using the same buffers as for the NA-electrophoretogram blot experiments, with 10^6 cpm of ick-translated cDNA per ml. After hybridization, the filter as washed twice for a total of 1 hr at room temperature in × SSC/0.1% $NaDodSO_4$. Then 1× SSC/0.1% $NaDodSO_4$ iat had been warmed to 68°C was added at room temperaire twice over 1 hr. Autoradiograms were exposed for 1–3 ays. Hybridization with a β-actin RNA probe was perormed to compare RNA loading. The levels of *mdr1* expreson were determined by densitometry of the autoradigrams.

RESULTS

he levels of *mdr1* mRNA were measured in samples of ormal tissues and various tumors as described in *Materials nd Methods*. A typical analysis of some normal tissues is nown in Fig. 2, and the results from a variety of experiments e summarized in Table 1. In each experiment, RNA from B lines with increased drug resistance and increased levels f *mdr1* mRNA were included so that the unknown samples ould be directly compared with samples of known *mdr1* NA content and known multidrug resistance. Relative to rug-sensitive KB-3-1, the multidrug-resistant subline KB-5 has a 40-fold increase in *mdr1* mRNA and KB-V1 (Vbl) as a greater than 500-fold increase. Adrenal tissue, and articularly adrenal medulla dissected free of most cortex,

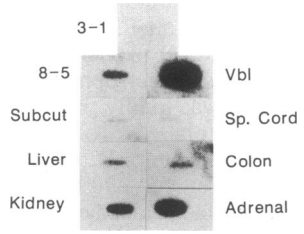

FIG. 2. *mdr1* expression in normal human tissues. Slot blot ybridization of RNA (10-μg samples) from selected tissues demnstrate the range of expression of *mdr1* RNA from low to high. bbreviations: 3-1, parental KB cell line; 8-5 and Vbl, multidrugsistant KB sublines; Subcut, subcutaneous tissue; Sp. cord, spinal ord.

Table 1. *mdr1* mRNA in normal tissues

Tissue or cell line*	mRNA level†
Adrenal (4)	160
Adrenal medulla	>500
Kidney (6)	50
Kidney medulla	75
Colon (10)	31
Liver (4)	25
Lung (9)	20
Jejunum	20
Rectum	20
Brain	12
Prostate	8
Skin, subcutaneous tissue, skeletal muscle, heart, spleen (2), bone marrow (3), lymphocytes, esophagus, stomach, ovary, kidney cortex, spinal cord	1–5
KB-3-1	1
KB-8-5‡	40
KB-V1 (Vbl)§	>500

*Number of tissue samples studied (when >1) is given in parentheses. Values given are means.

†Quantitated by densitometry of slot blots of 10 μg of total RNA. Values, expressed relative to level for drug-sensitive KB-3-1 cells, were determined by comparison with KB-8-5 RNA, which gave a reproducible, easily detectable signal.

‡Resistance relative to the parent KB-3-1 is 3-fold for doxorubicin and 6-fold for vinblastine.

§Resistance relative to the parent KB-3-1 is 420-fold for doxorubicin and 210-fold for vinblastine.

contained very high levels of *mdr1* mRNA (Figs. 2–4) and Table 1). The levels in kidney, and particularly kidney medulla, were also high. Colon, liver, lung, jejunum, and

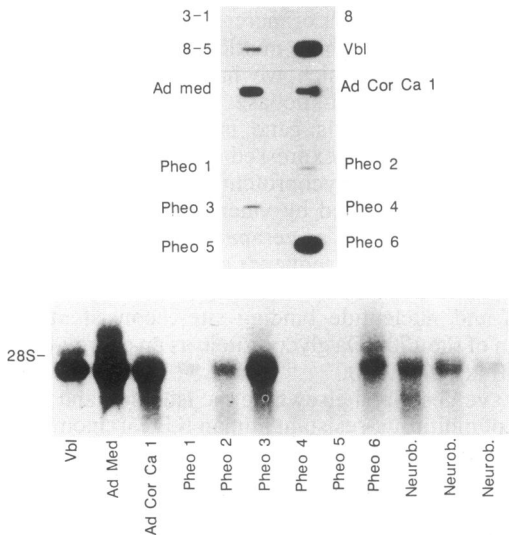

FIG. 3. *mdr1* expression in adrenal tissue and tumors. (*Upper*) Slot blot analysis. (*Lower*) Six micrograms of total RNA was electrophoresed in each lane before blot analysis, except for the Vbl and Pheo 6 lanes, which were loaded with 1 μg and 0.5 μg of total RNA, respectively. The three neuroblastoma (Neurob) samples were from patients who had failed treatment with combination chemotherapy that included vincristine and doxorubicin. Only the relevant portion of the autoradiogram is shown; position of 28S rRNA, which served as an internal marker, is indicated at left. Abbreviations: 3-1, parental KB cell line; 8, 8-5, and Vbl, multidrug-resistant KB sublines; Ad med, normal adrenal medulla; Ad Cor Ca, adrenocortical carcinoma; Pheo, pheochromocytoma.

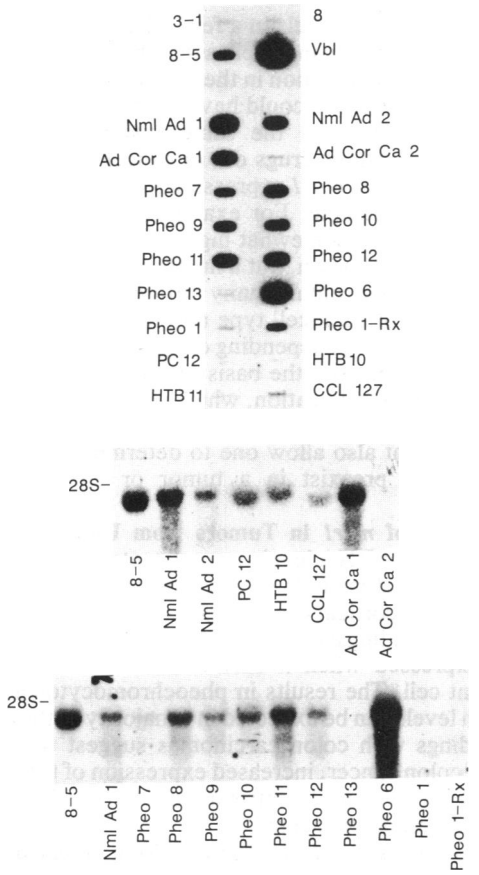

FIG. 4. *mdr1* expression in adrenal tumors and cell lines. (*Upper*) Slot blot analysis of levels of *mdr1* in normal adrenal (Nml Ad), tumors arising from the adrenal [adrenocortical carcinoma (Ad Cor Ca) and pheochromocytoma (Pheo)], and four cell lines of adrenal origin [PC12 (rat) and HTB 10, HTB 11, and CCL 127 (human)]. (*Lower*) Total RNA (8 μg per lane) was electrophoresed before blot-hybridization analysis. The figure is a composite of several gels and different autoradiographic exposures. Tissue for Pheo 1-Rx was obtained from an autopsy sample of Pheo 1, and the RNA had undergone partial degradation. On long exposures the 4.5-kb *mdr1* message could be visualized along with degraded products.

rectum had intermediate levels. The majority of organs and tissues had low levels, in the range of *mdr1* mRNA levels found in our most drug-sensitive (parental) cell line, KB-3-1.

The levels of *mdr1* mRNA were elevated in many tumors derived from the adrenal gland (Figs. 3 and 4 and Table 2). These included many pheochromocytomas, one adrenocortical carcinoma, and a few neuroblastomas. In all cases, a 4.5-kb mRNA species was detected, indicating that a similar mRNA was present in all the tumors. Both malignant and benign pheochromocytomas had increased *mdr1* mRNA levels. Of particular interest was pheochromocytoma 1, because samples of this malignant tumor were available before and after treatment with a regimen containing vincristine. The tumor initially responded but then became unresponsive. Levels of *mdr1* mRNA were increased 6-fold in the sample obtained at autopsy. Also shown in Fig. 3 are samples of three neuroblastomas from children who relapsed following combination chemotherapy that included vincristine and doxorubicin. These levels are higher than those measured in one untreated patient (data not shown) and in 12 neuroblastoma cell lines, three of which (HTB 10, HTB 11, and CCL 127) are shown in Fig. 4. Since most neuroblastoma cell lines have been established from untreated patients, it seems likely that low levels of *mdr1* mRNA are present in primary tumors. PC12, a rat pheochromocytoma line, had low levels of *mdr1* mRNA, as did normal rat adrenals (data not shown).

Table 2. *mdr1* mRNA in tumors

Tumor	Patient	Treatment*	mRNA level†
Pheochromocytoma	6		>500
	11		67
	12		64
	8		61
	9		40
	3		26
	10		19
	1	Vcr	18
	1		3
	7		15
	2		13
	4		1
	5		1
	13		1
Neuroblastoma	1	Vcr, Dox	25
	2	Vcr, Dox	24
	3	Vcr, Dox	10
Adrenocortical cancer	1		72
	2		2
Colon tumor‡	1		20 (42)
	2		23 (29)
	3	F-Ura, Mit C	54 (17)
	4		41 (21)
	5		20 (19)
	6		18 (31)
	7		76 (84)
	8		7 (6)
Childhood cancers			
Neuroepithelioma	1	Vcr, Act D	8
	2	Vcr, Act D	7
Ewing sarcoma	1	Vcr, Act D	1
	2	Vcr, Act D	7
Rhabdomyosarcoma	1	Vcr, Act D	7
	2	Vcr, Act D	74
Acute lymphoblastic leukemia	1–9		5–14
	10		68

*Some samples were from patients who had relapsed following combination chemotherapy, and some of the drugs these patients had received are shown: Vcr, vincristine; Dox, doxorubicin; F-Ura, 5-fluorouracil; Mit C, mitomycin C; Act D, actinomycin D.
†Relative to KB-3-1 cells. Data are from densitometry of blots shown in Figs. 3–7.
‡Values for adjacent normal colon tissue are given in parentheses.

Fig. 5 presents the results obtained in eight patients with primary colon carcinomas; *mdr1* mRNA levels in the tumors (T) or in nearby normal colon (N) are shown for each patient. Generally, normal colon and the tumors had *mdr1* mRNA levels in the intermediate range, although one set was in the high range and one was in the low range. No correlation could be established with the histology or degree of anaplasia. Two human colon carcinoma lines (WIDR and HTB 38) had low levels, and line LS-174 had intermediate levels (data not shown).

Figs. 6 and 7 present the results with samples obtained

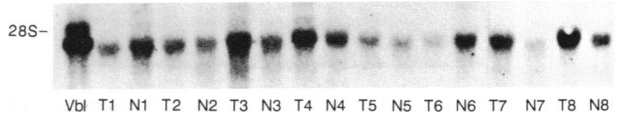

FIG. 5. *mdr1* expression in normal colon and colon tumors. Total (6 μg per lane) RNA from eight colon tumors (T) and corresponding adjacent normal (N) colon was electrophoresed before blot-hybridization analysis. The figure is a composite of three different gels, each with its own internal controls. Vbl, multidrug-resistant KB subline.

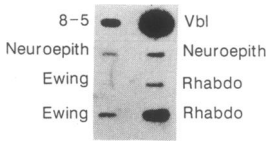

FIG. 6. *mdr1* expression in childhood cancers. Slot blot analysis RNA from six samples obtained at the time of relapse following initial chemotherapy. Abbreviations: 8-5 and Vbl, multidrug-resistant KB sublines; Neuroepith, neuroepithelioma; Ewing, Ewing sarcoma; Rhabdo, rhabdomyosarcoma.

from patients at the time of relapse following initial chemotherapy. All patients had received at least one of the drugs included in the multidrug-resistance phenotype. The slot blot shown in Fig. 6 consists of six samples obtained from children at the time of relapse. The level of *mdr1* RNA in one of the patients with a rhabdomyosarcoma is 10-fold higher than that of the other rhabdomyosarcoma. The level in skeletal muscle and in two other rhabdomyosarcomas was also low (data not shown). Although more extensive studies will be needed, this may be another example of an acquired increase in the level of *mdr1* message.

Fig. 7 shows blot-hybridization analysis of total RNA extracted from the leukemic cells of 10 patients with acute lymphoblastic leukemia, all of whom had >90% circulating lymphoblasts. The four lanes labeled I represent samples from patients at initial presentation. The lanes labeled R represent samples from patients that relapsed following combination chemotherapy that included vincristine and daunomycin. All four samples from the untreated patients and five of the six from the relapsed patients had low levels of *mdr1* RNA. However, one of the samples obtained from a patient at the time of a second relapse had levels that were 10-fold higher, raising the possibility that this increase had been acquired during drug therapy.

DISCUSSION

In the present study we have measured expression of a gene associated with multidrug resistance (*mdr1*) in many normal human tissues and human cancers. Our results show that the *mdr1* gene is expressed at a very high level in the adrenal gland; at high levels in kidney; at intermediate levels in lung, liver, jejunum, colon, and rectum; and at low levels in many other organs. We also found elevated *mdr1* expression in several human tumors. Many, but not all, tumors derived from the adrenal gland and the colon, organs with elevated *mdr1* mRNA levels, had high levels of *mdr1* expression. Increased levels were also observed in some tumors at the time of relapse following initial chemotherapy.

***mdr1* Expression in Normal Tissues.** The survey of normal tissues showed that *mdr1* is expressed at low levels in most organs and at higher levels in a few. The increased levels of expression in some organs implies that the *mdr1* gene product may have a normal function in these tissues. In kidney, liver, colon, or adrenal, *mdr1* could have a role in the excretion of organic molecules, and the same system could also be employed to transport drugs out of cancer cells.

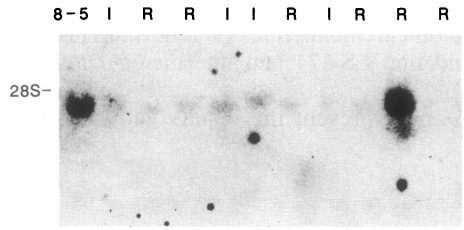

FIG. 7. *mdr1* expression in 10 samples of peripheral blood lymphocytes from patients with acute lymphoblastic leukemia. Total RNA (8 μg per lane) was electrophoresed before blot-hybridization analysis. Samples were obtained from patients at the time of initial presentation (I) or at the time of relapse (R) after treatment with regimens that included vincristine and daunomycin. Lane at left contained RNA from the multidrug-resistant line KB-8-5.

In normal tissues, *mdr1* expression was often found to vary from sample to sample. For example, of nine lung tissues examined, two had somewhat higher amounts. The basis of the variation is unknown, but a number of possibilities exist. One is that tissues contain many different types of cells and the proportion of each cell type may vary. A second is that individuals may vary, depending on genetic or environmental factors. To understand the basis of this variation, the technique of *in situ* hybridization, which allows measurements of RNA levels in individual cells, should be helpful. This technique might also allow one to determine whether drug-resistant cells preexist in a tumor or emerge following treatment.

Expression of *mdr1* in Tumors from Untreated Patients. Elevated *mdr1* mRNA levels were detected in tumors arising from the adrenal medulla (pheochromocytoma) and adrenal cortex (adrenocortical carcinoma), as well as in colon carcinomas. This finding indicates that the *mdr1* gene can continue to be expressed when a normal cell is converted into a malignant cell. The results in pheochromocytomas indicate that high levels can be expected in a majority of these tumors. Our findings with colon carcinomas suggest that, in some cases of colon cancer, increased expression of the *mdr1* gene might be involved in the intrinsic resistance of these tumors to chemotherapeutic agents.

Expression of *mdr1* in Tumors from Treated Patients. Increased levels of *mdr1* message were seen in some of our samples obtained from patients who relapsed after initial chemotherapy. Tissue from one pheochromocytoma was available before treatment and after therapy with a regimen including vincristine. After an initial response, the patient had progressive disease and died. The level of *mdr1* expression was increased at least 6-fold in the autopsy sample and may be an example of acquired multidrug resistance due to increased expression of the *mdr1* gene. This increased *mdr1* expression may represent reactivation of a previously active gene or selection of clones, initially present in the tumor, that contain higher levels of the *mdr1* message. High expression was also detected in neuroblastomas from patients who failed treatment with a regimen containing vincristine and actinomycin D. A sample from one untreated patient and many neuroblastoma cell lines from untreated patients show low *mdr1* expression (unpublished data). We also studied 10 patients with acute lymphocytic leukemia. One for whom treatment failed had distinctly elevated levels of *mdr1* mRNA. It is also of interest that one rhabdomyosarcoma from a patient treated with vincristine and actinomycin D had high *mdr1* mRNA levels, whereas three other rhabdomyosarcomas and normal skeletal muscle did not.

Control of *mdr1* Expression and Correlation of Levels of Expression with Drug Resistance. In cell lines selected for high levels of multidrug resistance, amplification of the *mdr1* gene is commonly observed (9). However, in mutants with lower levels of resistance, such as KB-8-5, we have detected increased *mdr1* expression without gene amplification. The levels of *mdr1* RNA detected in many tumors are comparable to KB-8-5 or lower and probably represent only increased *mdr1* transcription, suggesting that, in a clinical setting, gene activation might be a more common means of developing resistance than gene amplification.

The levels of *mdr1* RNA expression have been shown (9, 19) to correlate with the degree of drug resistance in human KB carcinoma cells with increased drug resistance. The parental cell line KB-3-1 is very drug-sensitive and expresses

a very low level of *mdr1* mRNA (which is arbitrarily set at 1). KB-8-5, which is 3 times as resistant to doxorubicin and 6 times as resistant to vinblastine, has an *mdr1* mRNA level around 40, and KB-V-1, which is 420 times as resistant to vinblastine and 210 times as resistant to doxorubicin, has an *mdr1* mRNA level >500. From the results presented here, we cannot identify a level of *mdr1* mRNA that confers drug resistance. However, the *mdr1* mRNA levels in the acute lymphoblastic leukemia samples were <10 in all but two cases. Acute lymphoblastic leukemia is a drug-sensitive malignancy, and our findings are in agreement with this observation.

Clinical Implications. It seems likely that more than one mechanism contributes to the process of multidrug resistance. The ability to identify a group of tumors that display multidrug resistance by measuring the levels of *mdr1* mRNA could have many important consequences. First, determination of the level of *mdr1* mRNA could allow the identification of tumors likely to fail to respond to a given drug. Clinical studies invariably include a fraction of patients with unresponsive tumors, with no clear explanation for why some patients fail while others respond. Identification of patients likely to fail could lead to the use of alternative treatment programs. Second, the level of *mdr1* mRNA could be used as a guide for the selection of drugs. For example, our data suggest that a vinca alkaloid, doxorubicin, or actinomycin D might not be effective in the therapy of pheochromocytoma. In a combination regimen, the lack of efficacy of these drugs would be obscured. Studies comparing regimens with and without these drugs would probably require a large number of patients in order to demonstrate statistical significance, and in a disease like pheochromocytoma, such a study might not be possible. A third and related use would be to assess recurrent tumors to determine whether resistance to a given agent has developed during initial therapy. This information could be used to identify those patients who might benefit from continued therapy with the same or similar drugs, as well as to select a group of patients who have developed resistance and for whom specific therapy might be considered. Drugs such as verapamil, diltiazem, and quinidine have been found to overcome multidrug resistance in cell culture and in some animal experiments (8, 21, 22). It would be of interest to use verapamil or a drug with similar action but fewer side effects in tumors that have high levels of *mdr1* expression. Possible candidates identified in this report include certain pheochromocytomas, adrenal carcinomas, and carcinomas of the colon. In addition, since the product of the *mdr1* gene appears to be a glycoprotein that is found in the plasma membrane, antibodies to this protein, either by themselves or in combination with toxins or radioisotopes, may be useful in selectively killing cancers containing high levels of this protein.

In conclusion, we have measured levels of *mdr1* mRNA in human tissues and human tumors because multidrug resistance in tissue culture systems is associated with elevated *mdr1* mRNA levels. We have found that *mdr1* mRNA is present at high levels in many untreated cancers of the adrenal and colon and in a few treated and unresponsive tumors of other organs. Although controlled clinical studies will be required, our results suggest that measurement of the level of *mdr1* mRNA may prove to be a valuable tool for guiding chemotherapy.

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